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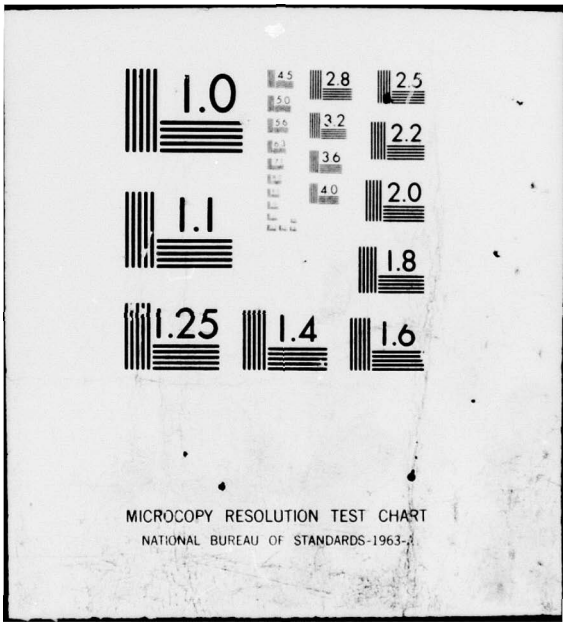
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LEVEL II

AVENUE TO UNDERSTANDING THE MECHANISM OF RADIATION EFFECTS . . .

J. Neyman

January 1980

STATISTICAL LABORATORY
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The visualized avenue towards understanding the michanism of the effects of radiation on the health of animals used in the experiments is, primarily, through willing interested cooperation between experimenting biologists, on the one hand, and equally willing and interested mathematical statisticians on the other.

A review of the developments over two recent decades leads the author to the

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20. ABSTRACT

following conclusions relating to experimental designs. (1) The design of survival experiments with serial sacrifices due to Arthur C. Upton is basic, but (2) This design needs an extension. The building of this extension depends very much on the inventiveness of experimenting biologists.

Avenue to Understanding the Mechanism of Radiation Effects
EXTENDED SERIAL SACRIFICE EXPERIMENTAL METHODOLOGY

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I. INTRODUCTION

The present paper was intended for delivery at the Workshop at the Institute for Energy Analysis, Oak Ridge Associated Universities, held in October 1979. I regret that I was not able to attend this Workshop. However, I did attend the earlier Workshop at the same Institute held in September 1977, the Proceedings of which have been just published [1]. I find these Proceedings very interesting, especially because of what I learned from experimenting biologists. This confirms my conviction that health effects of radiation is a highly interdisciplinary domain. Progress in this domain depends upon close collaboration between interested biologists-experimentors, on the one hand, and of equally interested statisticians, on the other. Here is an illustration.

There were 19 papers presented at the Workshop of 1977. Nine of them have titles including the words " competing risks." A substantial part of the statistical community is familiar with this term, particularly in connection with the "risk" of dying from this

or that specified "cause." E.g. in certain conditions thymic lymphoma is a "strong competitor" of reticulum cell sarcoma, etc.

Here, then, the term "competing risks" refers to phenomena supposed to develop in the bodies of living organism, the phenomena that are the subject of experimental and theoretical studies. However, when reading the contributions of P. J. Michael Fry, Everett Staffeldt and Sylvanus A. Tyler, [1, pp. 361-366], and of J. M. Holland, [1, pp. 367-370], I learned of a very different competition of risks. This competition is going on not in the bodies of experimental animals, but in the laboratories in which the dead animals are necropsied!

It appears that some diseases are easily detected by "gross examination," but some others are not and require the use of microscopes. Obviously, this kind of competition and its results are not helpful in the efforts to understand the mechanism of radiation effects on health and I am appreciative of the following insistence of Holland:

> "It is important to compare cohorts . . . with respect to specific disease states . . . it is essential that each animal receive the same qualitative and quantitative necropsy . . . fixed within examination protocal. Essential features of this protocal include a listing of all major organs and tissues . . ."

Hopefully, Dr. Holland's insistence in 1977 became a general rule in 1979. His "protocal" is mentioned in Chapter IV of this paper.

In my contribution to the Workshop of 1977, I expressed the view that the concept of "cause of death" is not useful and should

be abandoned. In consequence the many theories of competing risks became uninteresting. Realistic studies of health effects of exposure to any kind of possibly noxious agent depend upon the availability of data on "all major organs and tissues" as insisted by Holland.

The focus of the present paper is intended to be on happenings in the organisms of irradiated experimental animals. The thinking on this subject, my own and of other authors, evolved substantially during the years that elapsed. Some phases of this evolution are described below, including certain interesting experimental findings. My hope is that this description will create an appropriate perspective on the importance of the serial sacrifice methodology invented by Arthur C. Upton in 1969 [2].

II. EVOLUTION IN STUDIES OF HEALTH RELATED EFFECTS OF RADIATION

1. My first contact with the problem. My first contact with the problem of effects of irradiation on health goes back to the fall of 1958. In my capacity as a visitor at the National Institute of Health (NIH) I was asked to examine the literature relating to the existence of a threshold below which the irradiation could have no adverse health effects. At the time it was already commonly believed that adverse health effects of irradiation include cancer. The initiation of cancer was attributed to vaguely understood "mutations" supposed to occur in cells of living tissues.

Of the literature I read at the NIH the most relevant appeared two interconnected papers, both published in Science, Vol. 128 (1958). The first of the two papers, authored by M. P. Finkel (pp. 637-641)

describes experiments intended to estimate the irradiation threshold. The second paper, by A. M. Brues (pp. 693-699) describes theoretical reasons for the author's conviction that a threshold of irradiation carcinogenesis must exist. Briefly, Dr. Brues' reasons were that the mechanism of carcinogenesis must involve not just one but at least two stages, each involving mutations. Of these several stages, only the last is ascribed to irradiation. The earlier, the "precancerous stages" had to be ascribed to some other agents.

The reader will notice that the above description implies a ramification of the problem of carcinogenesis, irradiation -- yes, but in addition there had to be some other noxious agents, presumably chemical. In these circumstances it was natural for me to search for literature on experimental evidence of chemical carcinogenesis. This I found in several publications describing impressive experiments performed by M. B. Shimkin and M. J. Polissar, published in 1954, 1955 and 1958 in the Journal of the National Cancer Institute. The experiments were concerned with one particular chemical, the urethane.

The study of the Shimkin and Polissar experiments may be labeled as the second phase of my acquaintance with carcinogenesis. The third phase was especially concerned with one question posed by Dr. Brues, namely, whether the mechanism of carcinogenesis is one-stage or multi-stage. Influenced by the work of Shimkin and Polissar, the concern was with the urethane carcinogenesis (or "tumorogenesis") and, specifically, with the question of an experimental design that could provide an unambiguous resolution of the dilemma: one-stage or multi-stage?

The results of this study, comprising three interconnected papers, are published in the Proc. Fifth Berkeley Symposium Math.Stat. and Prob. Vol. 4 (1967) [Univ. of California Press, Berkeley, CA 94720]. The three papers in question were authored by Shimkin et al (pp. 707-720), by White et al (pp. 721-744) and by Neyman and Scott (pp. 745-776). The studies included a large number of experiments, conducted and analyzed in terms of contemporary biological "state-of-the-art", appeared consistent with the multi-stage hypothesis of carcinogenesis. It seemed appropriate to act on the assumption that the true mechanism of urethane tumorogenesis is a multi-stage mutation mechanism.

When I was writing the above conclusion, the old Latin saying came to my mind: "Oh fallacem hominem spem!" The disenchantment occurred a few years later when Margaret R. White completed her experiment intended to verify a particular assumption (or "presumption") of the earlier "state-of-the-art". Briefly and roughly, Margaret White used an innovative method to test two "classical" presumptions. One of them was that urethane injected into mice is eliminated from their bodies in a very short time, that, for practical purposes, could be considered instantaneous. The second classical presumption tested by Miss White was that the speed of eliminating urethane from the bodies of mice does not depend upon the dose injected. The innovative method of Miss White consisted in using "labeled" urethane.

The molecule of urethane includes three atoms of carbon. In her experiment Miss White used two kinds of labeled urethane, termed "C-labeled" and "E-labeled". In each case one particular atom of carbon was replaced by a radionucleide ^{14}C.

Miss White's results are published in two related papers, both in the Proc. of the Sixth Berkeley Symposium, Vol. 4 (1972) [Univ. of California Press, Berkeley, CA 94720]. The biological aspect of the study is described by Miss White (pp. 287-308). The part oriented towards statistical readers is in the paper of C. Guillier (pp. 309-316). The general conclusion of the two papers is that the speed of elimination of urethane from the bodies of mice depends strongly on the dose injected and, in consequence, that the mechanism of urethane tumorogenesis is likely to be a one-stage mechanism.

Some details of the above evolutionary phases will be found in the next chapter. As of now, it is appropriate to point out that the process of evolution was due to excessive reliance of particular research workers on preconceptions which changed from one phase to the next, when they were found unjustified. Compared to these, the serial sacrifice experimental methodology appears to depend on the least amount of preconceived ideas. Its aim is to find what are the facts. However, it does need certain extensions.

III. SOME DETAILS OF PARTICULAR EVOLUTIONARY PHASES.

(i) A. M. Brues. The following passage is reproduced from the article of Dr. A. M. Brues quoted earlier:

> There are many examples of induction of malignant disease through mechanisms which are clearly indirect--that is, where irradiation of a cell can be shown not to be the critical factor . . . There is a large body of evidence indicating that the malignant transformation occurs after a sequence of 'precancerous' stages has taken place. The most widely observed example is in the development of skin cancer, which, in whatever way it is produced, is likely to be preceded

by various types of benign atrophic or hyperplastic states; in experimental studies it most often develops in a benign papilloma.

(ii) M. B. Shimkin and M. J. Polissar. While the above quote illustrates indirect documentation of Dr. Brues, the following somewhat longish quote describes the Shimkin and Polissar efforts to obtain experimental evidence of the happenings in the lungs of mice following the injection of a single dose of urethane. The quote reproduces a passage in reference [3].

COUNTS OF CELLS, OF HYPERPLASTIC FOCI, AND OF TUMORS IN LUNGS OF MICE After Shimkin and Polissar [12].

Days after Urethane	*Estimated Mean Number of:* Cells per Square (106.3 sq. micra)	Presumed First Mutants per Square	Foci per Lung	Tumors per Lung
0	0.73	0.00	—	—
1	0.85	0.12	—	—
3	0.92	0.19	—	—
7	1.11	0.38	—	—
14	1.02	0.29	294	—
21	1.35	0.62	450	—
28	1.57	0.84	390	15.5
38	—	—	610	—
49	1.33	0.60	450	37.3
84	1.20	0.47	260	34.8
105	—	—	200	35.2
133	—	—	83	35.7

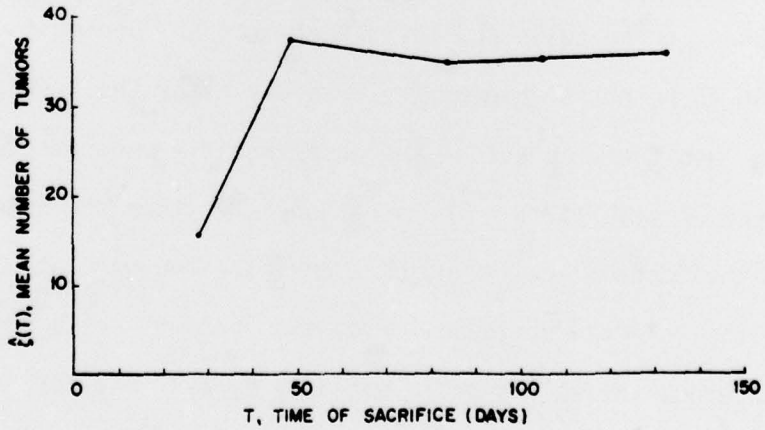


FIGURE 2

Estimated mean number of tumor nodules per lung. Each mouse received same dose of urethane 1 mg/gm BW, sacrificed at varying times T after injection.

> 8.1. Shimkin and Polissar data. Table I and figure 2 represent the experimental results of Shimkin and Polissar. For purposes of the present section, only the last column of the table is needed. This gives the estimated average number $\zeta(T)$ of tumor nodules in the lungs of mice all given the same dose of urethane 1 mg/gm BW, and sacrificed at varying times after the injection specified in the first column . . . Figure 2 shows a plot of $\zeta(T)$ against T. It is seen that, while the unavoidable random fluctuation of $\zeta(T)$ are quite noticeable, the convergence of $\zeta(T)$ to an asymptotic value is pronounced. Inspection of figure 2 suggests strongly that, if more than one series of mice were available, each series with a different dose D of urethane administered in a single injection, then counts of tumors made after some 20 weeks might reasonably be considered as empirical counterparts of $\zeta(+\infty)$, and used for the verification of the one stage mutation hypothesis.

The experimental data resulted from microscopic examination of slices of the lungs of mice -- a methodology that many other experimentalists did not use.

(iii) One-stage or multistage? The Shimkin and Polissar findings illustrated above refer to temporal changes in two characteristics of the lungs of mice: the abundance of presumed "first mutant" cells, including the "hyperplastic foci" and of tumors. The presumed first mutant cells and the foci increase up to about 49 days and then decline to zero. Thus, these cells and their foci must be "benign." The tumors begin to be noticeable on the 28th day after the urethane injection. Then their abundance increases rapidly and, after about 50 days, becomes stabilized. The question is whether the noted changes in the "benign" cells and of tumors are two unrelated consequences of the injected urethane, or do the "benign" cells represent the "precancerous" growth as anticipated by Brues? Another important question is: how to design an experiment that could answer the question unambiguously?

Regretfully, in their work just described, Shimkin and Polissar made their counts of the two kinds of objects independently from each other. Also the amount of time and labor was too high to repeat the experiment. The practical possibilities appeared limited to experiments with varying doses of urethane and with counts of tumors performed with naked eye as they appear on the surface of the lungs of the sacrificed mice. The basic idea of the many experiments actually performed was that, if the mechanism of carcinogenesis is one-stage, then the ultimate crop of tumors must be independent of the time pattern at which a given dose of urethane is injected, whether in a single injection or in several equal fractions administered a few days apart. On the other hand, if the true mechanism is multistage and if the interval between the successive injections happens to be close to the time of the maximum of "precancerous" cells, then the fractionated administration of a given dose would be expected to result in an increased number of tumors counted.

Briefly, the many experiments performed varied in many respects, including the strains of mice, some of which proved very variable in their response. However, one question appeared to have been answered unambiguously: The crop of tumors counted was found to depend sharply on the time pattern of administering the same total dose of urethane. Specifically, when a fixed total dose D of urethane, measured in milligrams per gram of body weight of the mice, was administered either at once or in equal fractions over about a month, the fractionated administration resulted in a very substantial DECREASE in the total number of tumors counted.

In accordance with the contemporary ideas about the effectiveness of urethane, the conclusion reached was that the mechanism of the urethane tumorogenesis cannot be a one-stage mechanism. The credit for questionning this conclusion and for performing an experiment to test one of its basic assumptions belongs to Margaret R. White.

(iv) Is the number of initial urethane tumorogenic events proportional to the dose injected? The two pages of graphs reproduced from the article of Guillier already quoted seem to be the most informative way to summarize the findings of Miss White.

The page on the left, Guillier's figures 2 and 3, illustrate Miss White's results obtained using the C-labelled urethane. The other page, with figures 4 and 5, refers to E-labelled urethane. The curves in all the figures, marked by different symbols, refer to five replicates of a specified experiment. In each case, the ordinate of a point on the curve represents the percentage of the total number of injected ^{14}C atoms that, after so many hours since the injection, still remain not removed from the bodies of the mice. At time zero, this percentage is 100%. Subsequently, as hours go by, the proportion of unremoved ^{14}C atoms decreases, first slowly, then faster, but eventually approaching a horizontal asymptote. The height of this asymptote reflects the proportion of the injected atoms ^{14}C that failed to be eliminated through the process studied, namely through exhaling. The remaining ^{14}C atoms may have been eliminated otherwise, perhaps in urine, or may have been incorporated in the bodies of mice, perhaps in their bones, etc.

The essential details of the four graphs are: (a) the ^{14}C atoms of the E-labelled urethane are exhaled more slowly than those of the

C-labelled urethane, which documents the fact that molecules of the urethane must have been metabolized, and (b) that the speed of the elimination of urethane is sharply dependent on the size of the dose injected. Figures 2 and 4 correspond to the dose of 1 mg/g of the body weight of the mice while Figures 3 and 5 correspond to the dose of one-eighth of one milligram.

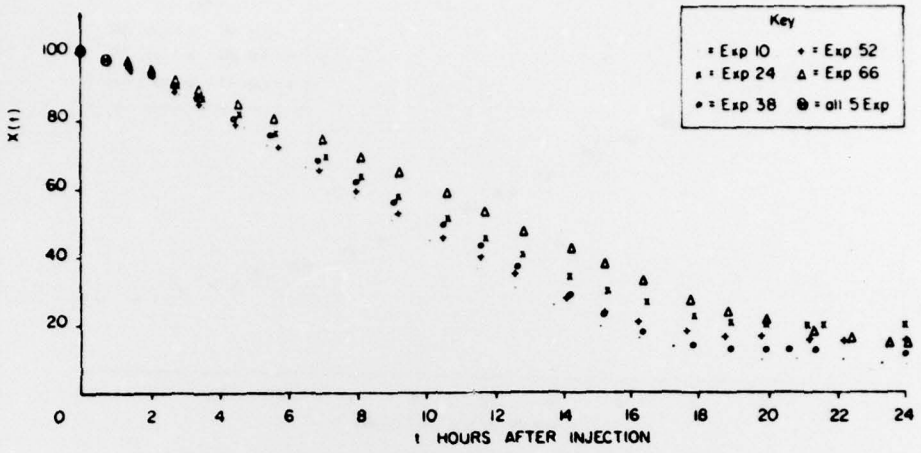
Note: Miss White's experiment included a substantial number of different dosages of urethane. The lowest was 0.125 mg/g. Next there were dosages 0.25 mg/g, 0.50 mg/g, 1.00 mg/g, 1.20 mg/g and 1.40 mg/g. With the two heaviest dosages some mice failed to survive the experiment. The results obtained with one-half and with one-quarter of a milligram were intermediate to those reproduced by Guillier, indicating the same general patterns.

The conclusion implied by Miss White's results is unambigious; with large doses of the injected urethane the entities within the bodies of mice that govern the exhaling (enzymes?) must have become temporarily overwhelmed. In other words, the molecules of the urethane (or their metabolites) spend more time in the bodies of mice if injected in larger doses and can produce relatively more "initial" tumorogenic effects than they could with small doses. This is confirmed by other details of Miss White's experiment.

What are the implications? What about the realism of the conclusion of the earlier phase that the urethane tumorogenesis cannot be one-stage?

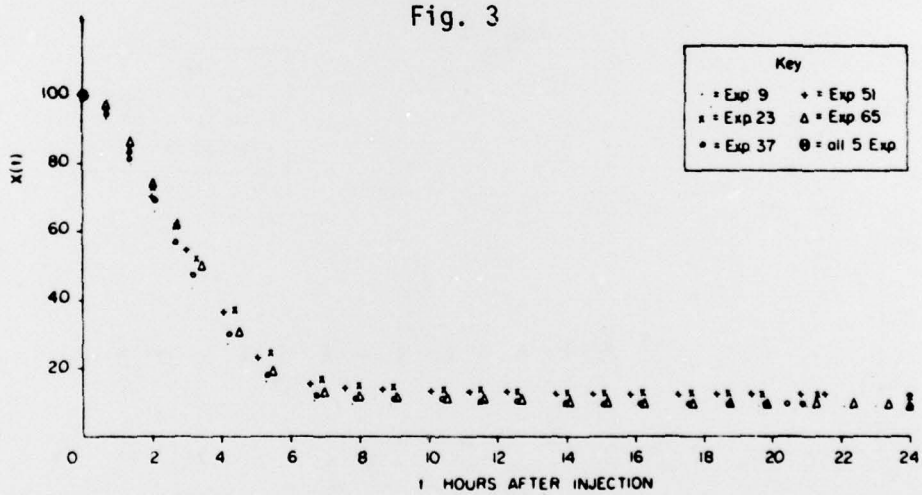
Margaret White's findings contradict this conclusion.

Fig. 2



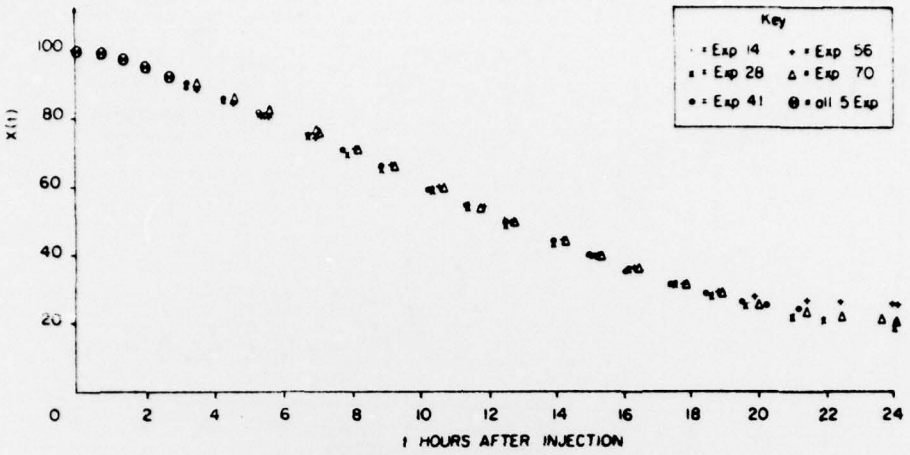
C LABELED URETHANE, DOSE 100 mg/g

Fig. 3



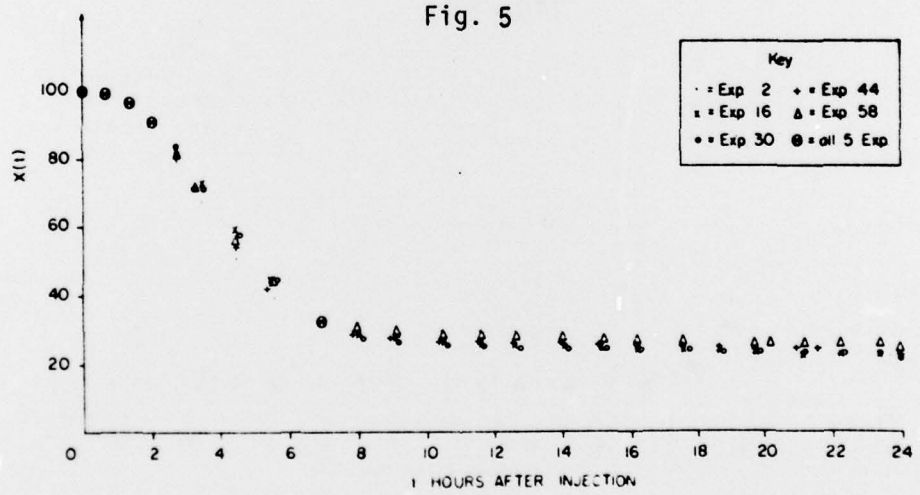
C LABELED URETHANE, DOSE 0.125 mg/g

Fig. 4



E LABELED URETHANE, DOSE 100 mg/g

Fig. 5



E LABELED URETHANE, DOSE 0.25 mg/g

IV. SERIAL SACRIFICE METHODOLOGY: A DIRECT APPROACH TO HEALTH EFFECTS OF IRRADIATION

(i) Analogy with insurance problems. The empirical study of effects of irradiation presupposes two groups of animals (say mice). One group is subjected to irradiation (say "experimentals") and the other is not (say "controls"). We have here an analogy with insurance related studies conducted for particular groups of the population, each group characterized by some specific conditions of life and employment. Such groups may be exemplified by school teachers, office workers and coal miners.

A question of interest may be whether a single set of acturial tables would be appropriate to use for all the three population groups mentioned. This would be the case if age dependent death and sickness rates for teachers, office workers and coal miners were approximately the same. Here, it is easy to guess that the rates for miners are likely to be different. But are they? And what about school teachers and office workers?

In order to answer these and many similar questions it is unavoidable to perform studies of "cohorts." Such studies are actually performed using the health and mortality records accumulated in various institutions such as clinics serving various occupational groups, etc. In order to be really reliable, the health records must conform with Dr. Holland's "protocol" (See Introduction) complete with all of its trimmings. A brief description of findings is as follows.

(ii) Stability periods. While particular occupational population groups exist with very different rates of death and sickness, the age related changes in these rates calculated for adults are relatively slow. For example, the year to year changes in death rates, etc. are

so small that some tables used in practice gave rates for periods of 5 years. In the following, periods of this kind, with negligible changes in the rates of interest, will be called "stability periods." The determination of the length of stability periods is made using empirical data and taking into account the purpose of the tables.

The reader will realize that such determination, as well as the definition of "adult ages" must be somewhat subjective.

(iii) Typical questions. For purposes of the present chapter it is very important to be clear about the typical questions that need to be answered in insurance related studies for specified occupational groups constructed for the adopted stability periods. Here are three questions.

Question 1 How frequently do coal miners die between the ages 40 and 45 while suffering from two specified diseases, say leukemia and influenza?

Question 2 How frequently are coal miners 40 years of age suffering from two diseases combined, namely from leukemia and influenza?

Question 3 refers to coal miners of the category specified in Question 2. Question 3 reads: how frequently do such coal miners die before reaching age 45? More generally, how frequently the coal miners of the same category change their health characteristics before they are 45, perhaps recovering from influenza or developing pneumonia, etc.?

Given good health services organized by the coal miners union, all these three questions can be answered without much trouble. The computable rates are used both for purposes of insurance and, not infrequently, for progress in medical diagnosis. Finally these rates are a "must" in the process of comparing danger in various fields

of employment. The reader will realize that just such rates are an emphatic MUST in an experimental study of irradiation effects on mice. The question is whether, and if so, what experimental design could provide Dr. Holland's protocol data for computing rates answering questions similar to the above questions 1, 2 and 3, this both for the experimental and for the control mice.

(iv) How to answer the three questions for mice? After establishing the stability periods for mice (and this may require some special experimentation), and granting a satisfactory observance of Holland's "protocol", questions of type 1 are easily answered.

One begins by sorting the mice, separately the experimentals and the controls, that are alive at the beginning of each stability period and count them separately for each period. During any given period some of the mice alive at the beginning will die. Thus, autopsies performed using Dr. Holland's protocol will indicate those having particular combinations of pathological characteristics. This will provide the data for computing the rates. With a detailed protocol, most of the rates will be zero.

Now, let us see whether strict adherance to Dr. Holland's protocol is sufficient to answer questions of type 2. In order to answer this question, one has to know the number of mice ALIVE at the beginning of a given stability period and, at that time, having a specified combination of pathological states. However, the establishment of any such combination of pathological states requires detailed autopsies. Obviously, in order to be able to answer the question of type 2, the only way is to take a sample of mice alive at the indicated moment, to "sacrifice" them and to perform the autopsies.

This has to be done for each adapted stability period. The necessity of such "serial sacrifices" has been first realized by Dr. Arthur C. Upton in 1969 [2]. It was Upton who organized a serial sacrifice experiment at the Oak Ridge National Laboratory. To begin with, the chief experimentor was Upton himself. Later, when Upton left the laboratory, the experiment was completed by Dr. John B. Storer.

(v) Question 3: an extension of the Upton methodology is needed. Question 3 refers to mice alive at the beginning of any stability period and having at that time a known set of pathological characteristics. The question asks for a documented prognosis of the changes in the health characteristics to be expected during the stability period in question.

The reader will realize that the serial sacrifice methodology does not provide data sufficient to formulate the prognosis. Consider a category of mice alive at the beginning of a stability period and having at that time some marks of "precancerous growth" of the kind discussed by Dr. Brues (See Chapter III). One of the possible changes in the state of health of such mice may be the initiation of a malignant growth, but not necessarily. The actual happenings can be observed by sacrificing a sample of the mice in question at the end of the stability period and by performing autopsies in accordance with the Holland protocol. However, in order to be able to compute the rate of interest, it is necessary to have a methodology for determining the frequency with which the mice alive at the beginning of the stability period are marked by the presence of "precancerous growth" without killing these mice.

The conclusion is that the Upton methodology needs an extension. A diagnostic procedure is needed with an efficiency at least approximating the protocol of Dr. Holland, but not requiring the killing or even hurting of these mice. Could something like Brues Ames' discovery be helpful?

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